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Highlights:

- We looked at the peripheral lymphocyte phenotypes in dog with babesiosis.
- The % of T lymphocytes were reduced in infected dogs versus controls.
- The % T helper lymphocytes was lower in complicated versus uncomplicated dogs.

ORIGINAL RESEARCH PAPER

A flow cytometric assessment of the lymphocyte immunophenotypes in dogs naturally infected with *Babesia rossi*

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ABSTRACT

Immunity to *Babesia* infection requires both innate and acquired responses, including cell mediated- and humoral responses. The aims of this study were to investigate the variation in selected peripheral blood lymphocyte phenotypes in dogs with virulent babesiosis at presentation and over time after treatment, and to determine whether these were correlated with the severity of clinical signs. Forty-four dogs naturally infected with *B. rossi* were studied and 5 healthy dogs were included as controls. Blood samples were collected from the jugular vein at admission, prior to any treatment, and at 24 hours and 48 – 72 hours. Leukocytes were incubated with canine specific, fluorochrome conjugated anti-CD3, anti-CD4, anti-CD8, and anti-B cell markers. *Babesia*-infected dogs were divided into complicated or uncomplicated groups on clinical grounds and in-house laboratory assays. The percentage $CD3^+$ lymphocytes in the complicated group was lower compared to the controls ($P = 0.014$) and uncomplicated group ($P = 0.007$). The percentage $CD4^+$ T lymphocytes in the complicated group was lower compared to the controls ($P = 0.027$) and uncomplicated group ($P = 0.014$). Both the complicated as well as the uncomplicated groups expressed a lower percentage $CD8^+$ T lymphocytes compared to the control group ($P<0.001$ and $P = 0.005$, respectively). The percentage B lymphocytes was higher in the complicated group at 48 - 72 hours. These findings could indicate the presence of a functional immune suppression secondary to increased apoptosis or redistribution of effector lymphocytes and/or a combination of other immune modulatory mechanisms induced by *B. rossi* infection.

Keywords: babesiosis, canine, flow cytometry, lymphocyte, immunology

1. Introduction

Babesiosis is the second most important hemoparasite in mammals (Schnittger et al., 2012), has a global distribution, causes significant animal morbidity and mortality, is an emerging zoonosis, and is the most important blood transfusion acquired disease in humans in the USA (Herwaldt et al., 2011; Krause et al., 2003). The dog parasite, *Babesia rossi*, is an intra-erythrocytic tick borne protozoan parasite that occurs predominantly in southern Africa (Schoeman, 2009). Of the six *Babesi*a parasite species that infect dogs, it is the most virulent (Schoeman, 2009). The infection results in a peracute as well as an acute disease and it is one of the most prevalent diseases in domestic dogs in South Africa, where it results in high morbidity and mortality (Collett, 2000). The severity of the disease has been reported to depend on the age and immune status of the host (Schoeman, 2009). Hemolytic anemia due to both intravascular and extravascular hemolyses is a consistent feature of the disease (Jacobson, 2006; Schoeman, 2009). Typical complications include severe anemia, cerebral pathology (characterized by seizures in normoglycemic dogs) (Jacobson, 2006), hepatopathy and icterus, secondary immune-mediate hemolytic anemia (IMHA), acute respiratory distress syndrome (ARDS) (Jacobson and Clark, 1994), hypoglycemia (Keller et al., 2004), acute kidney injury (AKI) (Jacobson and Clark, 1994; Lobetti and Jacobson, 2001), hyperlactatemia (Jacobson and Lobetti, 2005; Leisewitz et al., 2001; Nel et al., 2004), hemoconcentration (Jacobson and Clark, 1994; Welzl et al., 2001), pancreatitis (Mohr et al., 2000), and disseminated intravascular coagulation (DIC) (Goddard et al., 2013).

There is evidence to suggest that, similar to malaria, the disease caused by *Babesia* spp. is the result of an exuberant host response, which may lead to

systemic inflammatory response syndrome, collateral organ damage and even death (Ahmed, 2002; Clark and Jacobson, 1998; Hemmer et al., 2000; Welzl et al., 2001; Wright et al., 1989). Babesiosis and malaria share many similarities with regards to complex clinical signs, underlying pathophysiology and immunopathology with many of the disease features being attributed to the host response (Allred and Al-Khedery, 2004; Clark, 1982; Krause et al., 2007; Maegraith et al., 1957; Wright et al., 1988). Both diseases are vector borne and caused by an intra-erythrocytic protozoan (Krause et al., 2007). Much of the pathology associated with these diseases is thought to be secondary to a marked pro-inflammatory milieu resulting in organ failure and death (Clark and Jacobson, 1998; Goddard et al., 2016; Jacobson and Clark, 1994; Krause et al., 2007). The parallels between human malaria, canine babesiosis, sepsis, and other systemic inflammatory states are well recognized (Clark et al., 2004; Jacobson and Clark, 1994; Reyers et al., 1998). Severe systemic inflammation can also result in a hypo-inflammatory immune response (Bone et al., 1997; Hotchkiss and Karl, 2003; Hotchkiss et al., 2003). The complex interactions between pro- and anti-inflammatory responses have also been demonstrated in bovine babesiosis (Goff et al., 2001).

Immunity to *Babesia* infection depends on both innate and acquired responses, which are both T lymphocyte- and antibody-mediated (Brown, 2001). Resolution of acute *B. bovis* infection in immunologically naïve animals depends on a strong innate immune response that leads to activation of macrophages via interferon-γ (IFN-γ) and parasite derived products, and results in parasite elimination by phagocytosis and production of toxic macrophage metabolites, especially nitric oxide (Brown et al., 2006). Protection against clinical disease in persistently infected animals or in successfully immunized animals relies on activation of antigen-specific cluster of

differentiation (CD) 4^+ T lymphocytes (helper T lymphocytes [T_H]) that secrete IFN-γ and promote the production of neutralizing antibodies, which are critical to the adaptive immune response (Brown, 2001; Brown and Palmer, 1999; Goff et al., 1998). At present limited information on the host response to canine babesiosis is available. The *Babesia* parasite, more so after being damaged or inactivated by anti-babesial therapy, stimulates a rapid humoral antibody response, which enhances the cellular immune response (Adaszek et al., 2015; Wulansari et al., 2003). Clinical resolution of canine babesiosis has been suggested to be associated with the stimulation and intensity of the $CD4^+$ T lymphocyte cellular immunity (Adaszek et al., 2015).

The first objective of this study was to investigate the variation in selected peripheral blood lymphocyte phenotypes at presentation in dogs naturally infected with *B. rossi*, and to determine whether this was correlated with the severity of clinical signs. The second objective was to monitor the changes over time in the selected peripheral blood lymphocyte phenotypes after treatment. We hypothesized that the percentage of selected peripheral lymphocyte phenotypes would be decreased in dogs with the complicated form of the disease compared to dogs with the uncomplicated disease form and healthy controls.

2. Materials and methods

This was a prospective, descriptive longitudinal study, which included client-owned dogs, naturally infected with *B. rossi* that presented for veterinary care to the Onderstepoort Veterinary Academic Hospital, South Africa, between January 2014 and December 2014. The research protocol was approved by the University of Pretoria's Animal Ethics Committee (V091-13; 15 January 2014).

Infection with *Babesia* parasites was diagnosed by demonstration of intra-erythrocytic trophozoites on stained thin blood smears, and was confirmed as *B. rossi* by PCR and reverse line blot (RLB) (Matjila et al., 2008). The control dogs included five healthy, client-owned blood donor dogs. The control dogs were not matched for age or sex. Owner consent was obtained for enrolment of all the dogs in this study.

2. 1 Animals

Dogs diagnosed with babesiosis were included in the study if they fulfilled a defined set of selection criteria. Suitable dogs were of any breed and either sex, provided that they were ≥12 weeks of age, weighed ≥3 kg, and had a demonstrable parasitemia on a stained thin peripheral blood smear. Dogs were excluded if they were co-infected with *B. vogeli* or *Ehrlichia canis* based on PCR and RLB assay results. Dogs were also excluded if any signs of concurrent chronic or inflammatory disease conditions, any obvious infections or wounds, or any signs of trauma were present. Vaccination, glucocorticoid therapy, or any unrelated metabolic illness or babesiosis within the four weeks prior to presentation were also reasons for exclusion. Cases presenting with only mild or moderate anemia (hematocrit >12%), that were ambulatory and had no historical or clinical indication of significant concurrent organ involvement, were considered uncomplicated and were treated as outpatients. Dogs were classified with complicated disease when one or more of the following were identified: severe anemia (hematocrit ≤12%) necessitating transfusion with packed red cells; acute kidney injury (oliguria/anuria and persistently elevated serum creatinine concentration, despite appropriate fluid therapy); cerebral babesiosis (neurological signs in the face of normoglycemia and that could not be attributed to any other cause); hepatopathy and/or icterus (elevated alanine

aminotransferase and alkaline phosphatase activities with/without indications of cholestasis such as biliruburia and/or bilirubinemia; or elevated bile acids on a starved sample in the absence of any signs of cholestasis); hypoglycemia (blood glucose <3.3 mmol/L); hyperlactatemia (blood lactate >2.5 mmol/L); secondary immune-mediated hemolytic anemia (positive warm in-saline agglutination or Coombs-positive, and/or marked spherocytosis); acute respiratory distress syndrome (dyspnoea, adventitious lung sounds, radiological evidence of lung consolidation or edema, and blood-gas evidence of ventilation-perfusion mismatch); hemoconcentration (hematocrit >37% in association with intravascular hemolysis, low total protein, with concurrent clinical collapse); pancreatitis (elevated serum lipase activity, or a positive SNAP test for canine-specific pancreatic lipase (cPLI; IDEXX Laboratories) associated with abdominal pain, vomiting, ultrasonographic evidence of acute pancreatitis); and disseminated intravascular coagulation (thrombocytopenia with concurrent prolonged prothrombin and activated partial thromboplastin time, increased D-dimer and fibrin/fibrinogen degradation products concentrations, and decreased antithrombin activity).

The dogs received standard care for canine babesiosis, which included antibabesial treatment with diminazene aceturate (Berenil® RTU 0.07 g/mL, Intervet Schering-Plough Animal Health SA, Isando, Johannesburg, South Africa, dosed at 3.5 mg/kg once off), transfusion with packed red cells and intravenous fluids as needed. Dogs with secondary IMHA were treated with glucocorticoids (Lenisolone® 5mg, Aspen Pharmacare, Woodmead, Sandton, South Africa, dosed at 2 mg/kg per os twice a day). Additionally, any further related complications were treated accordingly at the discretion of the attending clinician. Control dogs were considered healthy based on history, a full clinical examination, peripheral blood smear

evaluation, complete blood count (CBC), as well as PCR and RLB assays to rule out parasitemia.

2. 2 Sample collection

Peripheral venous blood was collected at presentation prior to any treatment, and at 24 hours and 48 - 72 hours after presentation. Blood samples were collected from the jugular vein from each dog with a 21-gauge needle by careful venipuncture with minimal stasis. The blood samples were collected into serum and EDTA Vacutainer plastic tubes (BD Vacutainer tubes). The EDTA sample was used to perform a CBC, PCR and RLB assays, and flow cytometric evaluation.

2. 3 Reagents and antibodies for flow cytometric analysis

Red blood cell lysing solution (BD FACS Lysing Solution, catalog no. 349202, Franklin Lakes, New Jersey, USA), pH-balanced phosphate-buffered saline (BD CellWASH, catalog no. 349524, Franklin Lakes, New Jersey, USA) and calibration beads (SPHERO™ Rainbow Calibration Particles (6 and 8 Peaks), BD Biosciences, catalog no. 653144 and 653145, Franklin Lakes, New Jersey, USA) were used. The fluorochrome-labelled monoclonal antibodies for the surface markers of canine lymphocytes included APC-conjugated anti-Dog Pan T cell (Clone LSM 8.358; isotype mIgM) as the marker for T-lymphocytes; PE-conjugated anti-Dog CD4 (Clone LSM 12.125; isotype mIgG1) as the marker for $CD4^+$ T lymphocytes; FITC-conjugated anti-Dog CD8 (Clone LSM 1.140; isotype mIgG1) as the marker for $CD8^+$ T lymphocytes (Canine T Lymphocyte Cocktail, BD Biosciences, material number 558699, Franklin Lakes, New Jersey, USA); and PE-conjugated anti-B Cell marker (Clone LSM 11.425; isotype mIgG1) as the marker for B lymphocytes (Canine

Activated T Lymphocyte Cocktail, BD Biosciences, material number 558704, Franklin Lakes, New Jersey, USA) (Cobbold and Metcalfe, 1994).

2. 4 Sample preparation and staining method for identification of lymphocyte phenotypes

Based upon the peripheral lymphocyte count, a calculated volume of EDTA-anticoagulated whole blood, containing 10^6 lymphocytes, was centrifuged at 1520 *g* for 10 minutes at room temperature within one hour after blood collection to separate peripheral blood leukocytes (PBLs) from whole blood. This step ensured that enough leukocytes could be harvested, as well as effective lysis of the red blood cells in the sample tubes, as large volumes of blood were often required to obtain ~10^6 lymphocytes, because of peripheral leukopenia. The plasma was removed and 2 mL of BD FACS lysing solution was added to the cellular pellet and left for 15 minutes at room temperature to lyse all red blood cells after resuspending the cellular pellet. Thereafter the solution was centrifuged at 450 *g* for 20 minutes at 4˚C to concentrate the PBLs into a pellet, and the supernatant was removed with a pipette and sterile plastic tip. The pelleted PBLs were then resuspended and washed twice by the addition of 2 mL of cooled staining buffer (BD CellWASH) followed by centrifugation at 450 *g* for 20 minutes at 4˚C. PBLs, containing 10^6 lymphocytes, were then resuspended in 500 µL staining buffer, from which two tubes with 100 µL of the suspension were prepared and stained with 4 µL (20 µL of antibody per 10^6 cells) of the primary antibody mix (canine T lymphocyte cocktail; canine activated T lymphocyte cocktail) for 15 minutes in the dark at 4˚C. Once stained, the cells were washed once, as above, and resuspended in 500 µL staining buffer.

2. 5 Total white cell count and lymphocyte count determination and flow cytometric analysis

Lymphocyte counts were determined, within 30 minutes of collection, on the ADVIA 2120 (Siemens, Munich, Germany) through 2-dimensional laser light scattering. Flow cytometric analysis was performed by the primary author, within two to three hours of collection, on the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) interfaced with a computer and analysed using BD CSampler Software (BD Biosciences, Franklin Lakes, New Jersey, USA). The instrument was calibrated for sample acquisition using SPHERO™ Rainbow Calibration Particles (6 Peaks and 8 Peaks). Compensation was performed on all dual labelled samples to ensure adequate separation of events.

Unstained cells were used to define the lymphocyte population, based on their forward-angle and side-angle light scatter characteristics (Fig. 1a). Cells in the unstained lymphocyte gate were used to formulate the next plot and subsequently, the lymphocytes were gated based on their CD3 (APC), CD4 (PE), CD8 (FITC) or B cell marker (PE) expression (Fig. 1b – d). Cells that were dual positive for both CD3 and CD4 as well as CD3 and CD8 were defined as $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes, respectively. A minimum of 5000 lymphocytes were acquired for the initial gated population. Results were expressed as the percentage cells of the different phenotypes (T lymphocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes) within the lymphocyte population.

2. 6 DNA extraction and PCR

DNA was extracted from 200 µL EDTA-anticoagulated whole blood using a blood and tissue extraction kit (QIAmp blood and tissue extraction kit, Qiagen, Venlo, The

Netherlands) according to the manufacturer's instructions. Molecular diagnosis of *B. rossi* and exclusion of other *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* spp. were performed using PCR and RLB. PCR was conducted with a set of primers that amplified a 460-540 base pair fragment of the 18S SSU rRNA spanning the V4 region, a region conserved for *Babesia* and *Theileria*. The *Ehrlichia* PCR amplified the V1 hypervariable region of the 16S SSU rRNA. The membrane used for RLB included probes for *B vogeli*, *B. rossi*, *B. canis*, and *E. canis* (Matjila et al., 2008).

2. 7 Statistical analysis

The normality assumption was evaluated using the Shapiro-Wilk test and the total leukocyte and lymphocyte counts were log-transformed to approximate normality for between-group analysis. Age and weight were compared between the groups (complicated, uncomplicated and control) using analysis of variance (ANOVA) and gender proportions were compared using the Fisher's exact test. One-way ANOVA was used to assess differences between groups at presentation; if significant differences were present, the Bonferroni multiple comparison test was used as *post hoc* analysis. Differences between groups (complicated and uncomplicated) in the lymphocyte phenotypes over time was evaluated using linear mixed models with animal as random effect and time as the repeated measure, while adjusting for glucocorticoid therapy and packed red cell transfusion and applying the Bonferroni correction. Statistical analyses were performed using commercial software packages (SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, U.S.A), Stata 12.1 (StataCorp, College Station, TX, U.S.A.) and NCSS 2007 (NCSS, Kaysville, UT, U.S.A.)); $P \leq 0.05$ was considered significant.

3. Results

3. 1 Study population

Of the 48 dogs naturally infected with *Babesia* that were sampled, 44 dogs infected with *B. rossi* were included in the study: two dogs were co-infected with parvovirus, one dog was co-infected with *E. canis*, and one dog was only infected with *B. vogeli*. Twenty-two *Babesia*-infected dogs were included in each of the complicated and uncomplicated disease groups. Complications included: severe anemia (*n* = 11); AKI (*n* = 1); cerebral babesiosis (*n* = 1); hepatopathy and/or icterus (*n* = 11); hypoglycemia (*n* = 5); hyperlactatemia (*n* = 10); concurrent hypoglycemia and hyperlactatemia (*n* = 4); secondary IMHA (*n* = 8); ARDS (*n* = 2), and hemoconcentration (*n* = 2). The mean age (range) and weight (range) of the dogs in the complicated disease group were 26 months (3 - 91) and 16.5 kg (3.4 - 45.6), respectively, and included 17 male and five female dogs. The breeds included consisted of mixed breed (*n* = 8), Jack Russell terrier (*n* = 6), German shepherd dog (*n* = 3), Boerboel (*n* = 2), and one of each of the following, Siberian husky, chow chow, smooth-haired fox terrier, and American pit bull terrier. The mean age (range) and weight (range) of the dogs in the uncomplicated disease group were 27 months (4 - 84) and 13 kg (3.0 - 32), respectively, and included 12 male and 10 female dogs. The breeds included consisted of mixed breed (*n* = 8), Jack Russell terrier (*n* = 4), Labrador retriever (*n* = 3), and one of each of the following; basset hound, smooth-haired dachshund, wire-haired fox terrier, rottweiler, Yorkshire terrier, and cockerspaniel.

Five control dogs were sampled. The control dogs included two males and three females, with a mean age (range) and weight (range) of 52 months (24 - 76) and 28.2 kg (20.0 - 35.0), respectively. The breeds included German shepherd dog (n =

2), and one each of the following: standard French poodle, golden retriever, and a mixed breed.

The weight of the healthy control dogs was significantly higher (P = 0.011) compared to the uncomplicated disease group. There were no significant differences in age or gender between the three groups.

3. 2 Comparison of total leukocyte count, lymphocyte count and associated flow cytometric variables between groups at presentation.

Table 1 contains a summary of the variables for the groups at presentation. Results for 22 dogs in both the complicated and uncomplicated disease groups were available. There were no significant differences between groups in the mean total leukocyte count; however, compared to the controls, the mean lymphocyte count was significantly lower in both the complicated and uncomplicated disease groups (P = 0.008 and P = 0.003, respectively). The mean $CD3^+$ lymphocytes percentage in the complicated disease group was significantly lower compared to the controls (P = 0.014) and uncomplicated disease group (P = 0.007) (Fig. 2a). Similarly, the mean $CD4^+$ T lymphocytes percentage in the complicated disease group was significantly lower compared to the controls (P = 0.027) and uncomplicated disease group (P = 0.014) (Fig. 2b). Both the complicated and uncomplicated disease groups expressed a significantly lower mean $CD8^+$ T lymphocytes percentage compared to the control group (P< 0.001 and P = 0.005, respectively) (Fig. 2c); however, there was no significant difference between the two disease groups. There were no significant differences between the groups in the mean B lymphocytes percentage.

3. 3 Change over time in total leukocyte count, lymphocyte count, and lymphocyte phenotypes, and differences between groups at, 24 and 48 - 72 hours post-presentation

Table 2 contains a summary of the variables for the complicated and uncomplicated disease groups at 24 hours as well as 48 - 72 hours post-presentation. Results of 18 dogs in both the complicated and uncomplicated disease groups were available for the 24 hour post-presentation period, and results of 13 dogs in both the complicated and uncomplicated disease groups were available for the 48 - 72 hours post-presentation period. The mean total leukocyte count was significantly increased at 24 hours and 48 - 72 hours compared to the values at presentation for the complicated ($P = 0.016$ and $P = 0.002$, respectively) and uncomplicated ($P < 0.001$ for both) disease groups. Similarly, the mean lymphocyte count was significantly increased at 24 hours and 48 - 72 hours compared to the values at presentation for the complicated ($P < 0.001$ for both) and uncomplicated ($P < 0.001$ for both) disease groups. Treatment with packed red blood cells or daily glucocorticoids post-presentation did not have any significant effect on the total leukocyte or lymphocyte counts.

Evaluation of the mean $CD3^+$ T lymphocyte phenotype percentages for the complicated and uncomplicated disease groups at 24 hours and 48 - 72 hours revealed no significant difference between groups or over time. The mean $CD4^+$ T lymphocyte percentage was significantly decreased in the uncomplicated disease group at 48 - 72 hours ($P = 0.019$). The mean $CD8^+$ T lymphocyte percentage was significantly increased in the uncomplicated disease group at 24 hours ($P = 0.047$). In addition, the mean $CD8^+$ T lymphocyte percentage was significantly higher in the uncomplicated disease group compared to the complicated disease group at 24

hours (P = 0.014) (Fig. 2d). The mean B lymphocyte percentage was significantly increased in the complicated disease group at 48 - 72 hours (P = 0.041). Treatment with packed red blood cells had no effect on any of the lymphocyte phenotype variables; however, dogs treated with daily glucocorticoids post-presentation had a significantly higher percentage of B lymphocytes (P = 0.020) at the time points post-presentation.

4. Discussion

This is the first report of a reduced percentage of T lymphocytes ($CD3^+$), specifically $CD4^+$ and $CD8^+$ T lymphocytes, in the peripheral blood of dogs infected with *B. rossi*. In addition, the study also showed that the percentage of T lymphocytes in dogs with the complicated form of the disease was significantly lower compared to dogs with the uncomplicated form of babesiosis. A significant reduction in the percentage of the T_H lymphocytes ($CD3^+$ $CD4^+$) was evident in the complicated *Babesia*-infected dogs compared to both the healthy control dogs and uncomplicated *Babesia*-infected dogs. Although other studies have reported on similar decreases in the percentage and number of T lymphocytes and T_H lymphocytes in peripheral blood of dogs naturally infected with *B. canis* (Adaszek et al., 2015) and experimentally infected with *B. gibsoni* (Wulansari et al., 2003), the host immune response to *Babesia* infection has not been extensively studied. The reduced percentage of T lymphocytes, especially T_H ($CD4^+$) lymphocytes, at presentation in dogs infected with *B. rossi* may be indicative of a functional immunosuppression. Similarly, the lymphoproliferative response in dogs naturally infected with *B. gibsoni*, in particular those with subclinical infections or relapse infection, revealed depression of lymphocyte blastogenesis (Adachi et al., 1993).

In malaria, T lymphocytes dictate the magnitude of the parasite load entering the erythrocytic cycle by directly controlling the growth and development of the pre-erythrocytic *Plasmodium* stages, as well as the pathogenesis of the infection by directing the immune response elicited by the erythrocytic stage (Spence and Langhorne, 2012). Moreover, during the erythrocytic life cycle stages, $CD4^+$ T lymphocytes also contribute to parasite elimination through cytokine secretion, macrophage activation and the direction of humoral immunity (Pombo et al., 2002; Spence and Langhorne, 2012). In the erythrocytic life cycle stages it is assumed that humoral immune responses play a key role in host response (Langhorne et al., 2008). In murine models B lymphocytes and antibodies are critical in eliminating parasites and also play a regulatory role in $CD4^+$ T lymphocyte subset responses (Langhorne et al., 1998).

A reduction in the percentage of $CD4^+$ T lymphocytes was noted in the uncomplicated *Babesia*-infected dogs at 48 - 72 hours post-presentation compared to the percentage at presentation. In contrast, the percentage of $CD4^+$ T lymphocytes was not significantly different at either time point post-infection compared to the percentage at presentation in the complicated *Babesia*-infected dogs. These findings differ from a recent study that showed an increased percentage of $CD4^+$ T lymphocytes after treatment with imidocarb in dogs naturally infected with *B. canis* (Adaszek et al., 2015). However, that study did not differentiate between complicated and uncomplicated cases. Moreover, the infected dogs in our study were treated with diminazene aceturate, a minor-groove-binding agent that acts preferentially at the level of the mitochondrial DNA, thus influencing cell proliferation, ultrastructure, and mitochondrial activity of the targeted parasite (Shapiro and Englund, 1990). In addition to its anti-parasitic effect, it has been

shown that diminazene aceturate administration during infection also modulates the host immune response by diminishing T lymphocyte and macrophage hyperactivation, lowering the percentage expression of FoxP3 regulatory T lymphocytes, as well as lowering the systemic pro-inflammatory cytokine concentrations (Kuriakose et al., 2012). We suggest that, in our population of dogs, the combination of infection with *B. rossi* (which reduces the proportion of T_H lymphocytes), together with the reported immunosuppressive effect of diminazene aceturate, resulted in host immunosuppression. *B. canis* and *B. gibsoni* are also less virulent parasites and hence may not have the same effect on the immune system as *B. rossi*.

In our study the percentage of $CD8^+$ T lymphocytes was significantly reduced in both the complicated and uncomplicated *Babesia*-infected dogs compared to the healthy control dogs at presentation. Previous studies have reported that $CD8^+$ T lymphocytes do not seem to play a role in parasite clearance during the acute phase of *Babesia* spp. infections (Igarashi et al., 1999; Shimada et al., 1996). In murine malaria models $CD8^+$ T lymphocytes producing IFN-γ are crucial for host protection during the pre-erythrocytic life cycle stage (Schofield et al., 1987). In mice infected with *P. yoelii*, lower numbers of effector CD8+ T lymphocytes were found to be mainly due to increased apoptosis rather than reduced recruitment or proliferation rates of naïve T lymphocytes (White et al., 2015). Similar mechanisms resulting in slower expansion kinetics of antigen-specific $CD8^+$ T lymphocytes may be involved in *B.rossi*-infected dogs. Furthermore, unlike malarial parasites, *Babesia* spp. do not have a tissue stage in the mammalian host which may also account for the lack of requirement of $CD8^+$ T lymphocytes in the immune response against the early phase of the infection (Brown, 2001).

The percentage of $CD8^+$ T lymphocytes was significantly higher in the uncomplicated *Babesia*-infected dogs at 24 hours post-presentation. Similar findings have been reported in dogs infected with *B. canis* and *B. gibsoni* (Adaszek et al., 2015; Wulansari et al., 2003). In *Babesia* infections the central role of $CD4^+$ T lymphocytes is to aid in cell mediated immunity, including the proliferation of $CD8^+$ T lymphocytes, activation of macrophages, or the production of cytokines, as well as the humoral immune response (Preston and Jongejan, 1999). Therefore, the reduced percentage of $CD8^+$ T lymphocytes at 24 hours post-presentation in the complicated *Babesia*-infected dogs compared to uncomplicated *Babesia*-infected dogs may be as a result of the significantly reduced proportion of $CD4^+$ T lymphocytes in the complicated *Babesia*-infected dogs at presentation.

Compared to the uncomplicated group, the complicated *Babesia*-infected dogs had a significantly reduced percentage of circulating T lymphocytes, in particular T_H lymphocytes. Dogs with complicated disease have a reported mortality rate of 10%, with 80% dying within the first 24 hours of hospital admission (Keller et al., 2004; Nel et al., 2004) (Schoeman et al., 2007). Increased concentrations of pro-inflammatory cytokines, in particular interleukin-6 and monocyte-chemotactic protein-1 (Goddard et al., 2016), and a high parasitemia (Bohm et al., 2006) have been associated with increased mortality. The lower proportion of circulating lymphocyte subtypes in the complicated disease group in our study suggests a specific profile of inflammatory and modulatory cytokines, potentially related to a higher parasite burden, resulting in a unique host immune response in this particular group of *Babesia*-infected dogs.

No significant differences were noted at presentation in the percentage of B lymphocytes in the *Babesia*-infected dogs and the healthy control dogs. This is similar to what has been reported in dogs infected with *B. canis* (Adaszek et al.,

2015). This is not an unexpected finding since it is doubtful that antibody-dependent mechanisms of immunity play a role in the first few rounds of parasite multiplication within an infection. However, it has been shown that antibody mediated neutralization does play a role in the short period between the tick bite and erythrocyte invasion during which time the *Babesia* parasite is extracellular and exposed (Abdalla et al., 1978; Salama et al., 2013). Contrary to the group of dogs infected with *B. canis*, where the percentage of $CD21^+$ B lymphocytes remained unchanged throughout the entire study period (Adaszek et al., 2015), the percentage of B lymphocytes in our study increased at 48 - 72 hours post-presentation in the complicated *Babesia*-infected group. In dogs infected with *B. gibsoni*, the $CD21^+$ B lymphocytes in treated animals showed the first significant increase 14 days after infection, which corresponded to 7 days after initiation of clindamycin therapy (Wulansari et al., 2003). The findings of an upregulation of T helper response, accompanied by an IgG response, in mice infected with *B. microti* (Chen et al., 2000), suggest that, similar to malaria, antibody responses are crucial for controlling parasitemia after the acute infection is resolved (Fell and Smith, 1998; Taylor-Robinson, 1995). Therefore, the increased percentage B lymphocytes in the *Babesia*-infected dogs may be representative of a later T_H2 aided B-cell-dependant type response.

In general, the effects of glucocorticoids on lymphocytes are variable and dependent on the glucocorticoid dose, as well as on the lymphoid subpopulation and activation state (Ammersbach et al., 2006). A significant decrease in the number of circulating B lymphocytes in dogs treated daily for 14 days with prednisone has been reported (Rinkardt et al., 1999). In addition, immunosuppressive dosages of prednisone given to dogs for three consecutive days resulted in marked decreases in

the percentage of lymph node $CD21^+$ B lymphocytes at day 1, 3 and 38 after initiation of therapy (Ammersbach et al., 2006). In our study we observed that *Babesia*-infected dogs that received glucocorticoids showed a significantly higher percentage circulating B lymphocytes at the time points post-presentation compared to *Babesia*-infected dogs that did not received glucocorticoid therapy. The subset of dogs that did receive glucocorticoid therapy in our study were all classified as having secondary IMHA as a complication (n = 8). It therefore appears that the increase in the percentage of B lymphocytes noted in the dogs that received glucocorticoid therapy, may be attributed directly to increased antibody production in IMHA, or it may be as a result of variation in the B lymphocyte response to glucocorticoids in infected dogs with an associated secondary IMHA. It may also represent a delayed effect of glucocorticoids on the B lymphocyte population. Interestingly, no statistically significant effect of glucocorticoid therapy was noted on the T lymphocytes in our study. This is in contrast to a reported decrease in circulating $CD4^+$ and $CD8^+$ T lymphocytes in healthy dogs receiving prednisone at a daily dosage of 2 mg/kg for 14 days (Rinkardt et al., 1999). Prednisone has been reported to induce *in vitro* apoptosis of peripheral T lymphocytes in a time- and dose-dependent fashion (Lanza et al., 1996). The lack of a glucocorticoid effect noted on the T lymphocytes and subsets in our study may reflect either a delayed effect of glucocorticoids, or that the dosage used was not adequate to induce a decrease in the circulating T lymphocytes.

Although the mean blood lymphocyte count for both uncomplicated and complicated *Babesia*-infected dogs was within the laboratory reference interval (1 – 4.8 $x10^9$/L) throughout the study, similar to a previous study of dogs infected with *B. rossi* (Scheepers et al., 2011), it was significantly lower in the group of infected dogs

compared to the healthy control dogs. The lymphocyte count also increased significantly at 24 hours and 48 – 72 hours post-presentation in both the complicated and uncomplicated disease groups compared to values at presentation. Lymphocyte migration and retention in several organs, especially the spleen and liver, have been demonstrated during peak parasitemia in murine *P. chabaudi* infections (Kumararatne et al., 1987; Playfair and de Souza, 1982). Furthermore, the observed peripheral leukocytosis (primarily due to a lymphocytosis) two to five days after peak parasitemia in murine malaria was determined to be due to a redistribution of the lymphocyte pool (Kumararatne et al., 1987). Redistribution of splenic cells is central to the acute immune response of naïve animals to hemoparasitic infection and remarkable similarities of this acute splenic response have been demonstrated in both *B. bovis*-infected calves (Schneider et al., 2011) as well as *P. chabaudi*-infected mice (Leisewitz et al., 2004). Investigation of the lymphocyte trafficking patterns in canine babesiosis might provide valuable insights into the protective role of the spleen against babesiosis.

Limitations to this study included its clinical nature which prohibited standardization of the time period of infection prior to presentation; dogs were therefore at different stages of infection when presented, which could have influenced the results. Another limitation is the lack of specific isotype controls to define fluorescence gates for the control population and to detect the presence of non-specific staining. Furthermore, antibody saturation curves were not performed to determine the optimal concentration to use for each sample; nonetheless, the concentration of lymphocytes in each sample was known which allowed the calculation of an appropriate volume of antibody based on the manufacturer's recommendation.

5. Conclusions

This study has demonstrated a significantly reduced percentage of both $CD4^+$ and $CD8^+$ T lymphocytes in the peripheral blood of dogs naturally infected with *B. rossi*, particular in the dogs with the complicated form of the disease. These findings are consistent with the presence of a functional immune suppression secondary to increased apoptosis or redistribution of effector T lymphocytes and/or a combination of other immune modulatory mechanisms caused by *B. rossi* infection. Our results suggest that additional studies are required to further elucidate the immune response caused by *B. rossi*-infection, particularly in the spleen. A hypo-inflammatory immune response might be just as detrimental as an excessive systemic inflammatory response. A better understanding of the canine host immune response to *Babesia* infection may aid in vaccine development as well as immunotherapeutic approaches tailored to the individual immune status, resulting in appropriate regulation of the immune response which might be the key to improved outcomes in these dogs.

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Figure legends

Fig. 1 Flow cytometric analysis of peripheral lymphocyte phenotypes. (a) The lymphocyte population was identified and gated based on forward-angle and side-angle light scatter characteristics of unstained cells. (b – d) Subsequently the lymphocytes were gated on their CD3 (in APC) (b), B cell marker (in PE) (b), CD4 (in PE) (c), or CD8 (in FITC) (d) expression. Values in the quadrant indicate the percentage of cells.

Fig. 2 Box plots of the $CD3^+$ lymphocyte (a), $CD4^+$ T lymphocyte (b), and $CD8^+$ T lymphocyte (c) percentages in the healthy control group of dogs and the *Babesia*-infected dogs with uncomplicated and complicated disease at admission, and the 24 hours post-presentation $CD8^+$ lymphocyte percentages in the *Babesia*-infected dogs with uncomplicated and complicated disease (d). The box represents the interquartile range (IQR), the line represents the median, the upper and lower whiskers represent values within 1.5×IQR of the 75^{th} and 25^{th} percentiles respectively; outliers are plotted as open circles and asterisks represent extreme outlier values that are 3 times removed from the IQR.

Table 1 Hematologic and lymphocyte phenotype data in healthy control dogs and in dogs with complicated and uncomplicated babesiosis at presentation.

Variable	Controls (*n* = 5)	*Babesia*-infected (*n* = 44)	Complicated *Babesia*-infected (*n* = 22)	Uncomplicated *Babesia*-infected (*n* = 22)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total white cell count (x10^9/L)	10.08 ± 2.70	9.32 ± 15.20	12.46 ± 21.06	6.18 ± 2.98
Lymphocyte count (x10^9/L)	2.71 ± 1.07 [a,b,c]	1.33 ± 0.80 [a]	1.41 ± 1.0 [b]	1.27 ± 0.61 [c]
$CD3^+$ lymphocytes (%)	65.24 ± 15.33 [b]	43.64 ± 24.70	32.96 ± 23.04 [b,d]	54.32 ± 21.90 [d]
$CD3^+/4^+$ lymphocytes (%)	40.56 ± 15.38 [b]	26.76 ± 16.57	19.91 ± 15.37 [b,d]	33.61 ± 15.10 [d]
$CD3^+/8^+$ lymphocytes (%)	21.62 ± 17.27 [a,b,c]	8.10 ± 5.40 [a]	6.25 ± 5.58 [b]	9.95 ± 4.64 [c]
B-cell lymphocytes (%)	9.22 ± 4.00	6.16 ± 5.10	4.88 ± 3.28	7.44 ± 6.19

[a] Significant difference between control and *Babesia*-infected group ($P < 0.05$)

[b] Significant difference between control and complicated *Babesia*-infected group ($P < 0.05$)

[c] Significant difference between control and uncomplicated *Babesia*-infected group ($P < 0.05$)

[d] Significant difference between complicated *Babesia*-infected group and uncomplicated *Babesia*-infected group ($P < 0.05$)

Table 2 Hematologic and lymphocyte phenotype data in dogs with complicated and uncomplicated babesiosis at 24 hours and 48 - 72 hours post-presentation.

Variable	24 hours		48 - 72 hours	
	Complicated (*n* = 18)	Uncomplicated (*n* = 18)	Complicated (*n* = 13)	Uncomplicated (*n* = 13)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total white cell count (x10^9/L)	15.68 ± 12.11 [a]	12.09 ± 5.63 [c]	19.94 ± 9.27 [b]	18.25 ± 10.20 [d]
Lymphocyte count (x10^9/L)	2.35 ± 1.20 [a]	3.53 ± 1.88 [c]	3.33 ± 1.43 [b]	4.63 ± 3.61 [d]
$CD3^+$ lymphocytes (%)	40.44 ± 16.96	50.31 ± 17.63	45.57 ± 12.52	42.88 ± 19.02
$CD3^+/4^+$ lymphocytes (%)	24.98 ± 11.07	30.12 ± 12.26	26.51 ± 9.22	25.11 ± 9.49 [d]
$CD3^+/8^+$ lymphocytes (%)	8.12 ± 6.06 [e]	13.02 ± 7.28 [c,e]	8.18 ± 3.64	12 ± 7.71
B-cell lymphocytes (%)	6.64 ± 7.12	6.57 ± 5.46	12.64 ± 9.70 [b]	9.90 ± 6.40

[a] Significant difference between complicated *Babesia*-infected group at presentation and 24 hours post-presentation ($P < 0.05$)

[b] Significant difference between complicated *Babesia*-infected group at presentation and 48 - 72 hours post-presentation ($P < 0.05$)

[c] Significant difference between uncomplicated *Babesia*-infected group at presentation and 24 hours post-presentation ($P < 0.05$)

[d] Significant difference between uncomplicated *Babesia*-infected group at presentation and 48 - 72 hours post-presentation ($P < 0.05$)

[e] Significant difference between complicated *Babesia*-infected group and uncomplicated *Babesia*-infected group at specified time point ($P < 0.05$)

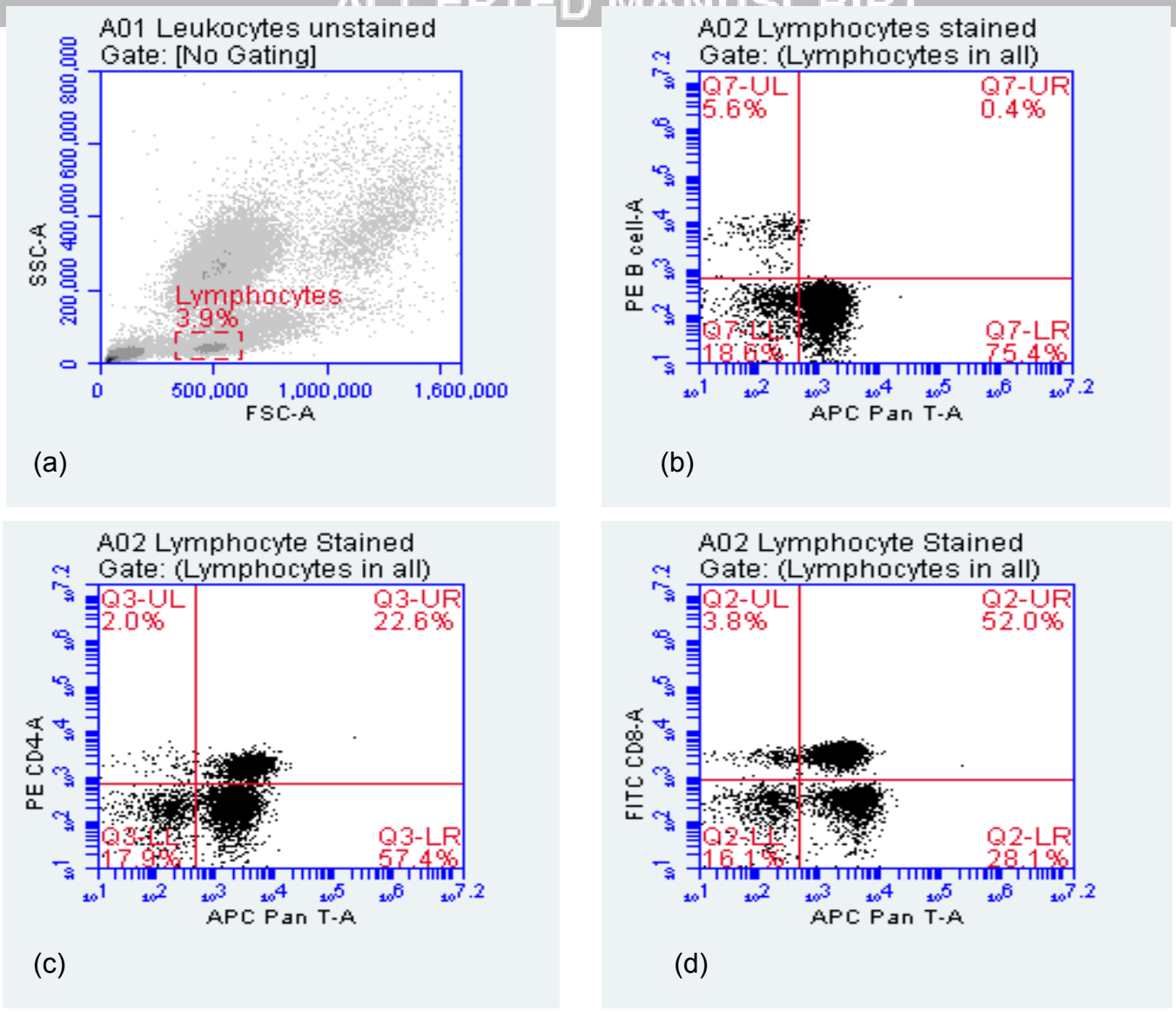

Fig. 1 Flow cytometric analysis of peripheral lymphocyte phenotypes. (a) The lymphocyte population was identified and gated based on forward-angle and side-angle light scatter characteristics of unstained cells. (b – d) Subsequently the lymphocytes were gated on their CD3 (APC) (b), B cell marker (PE) (b), CD4 (PE) (c), or CD8 (FITC) (d) expression. Values in the quadrant indicate the percentage of cells.

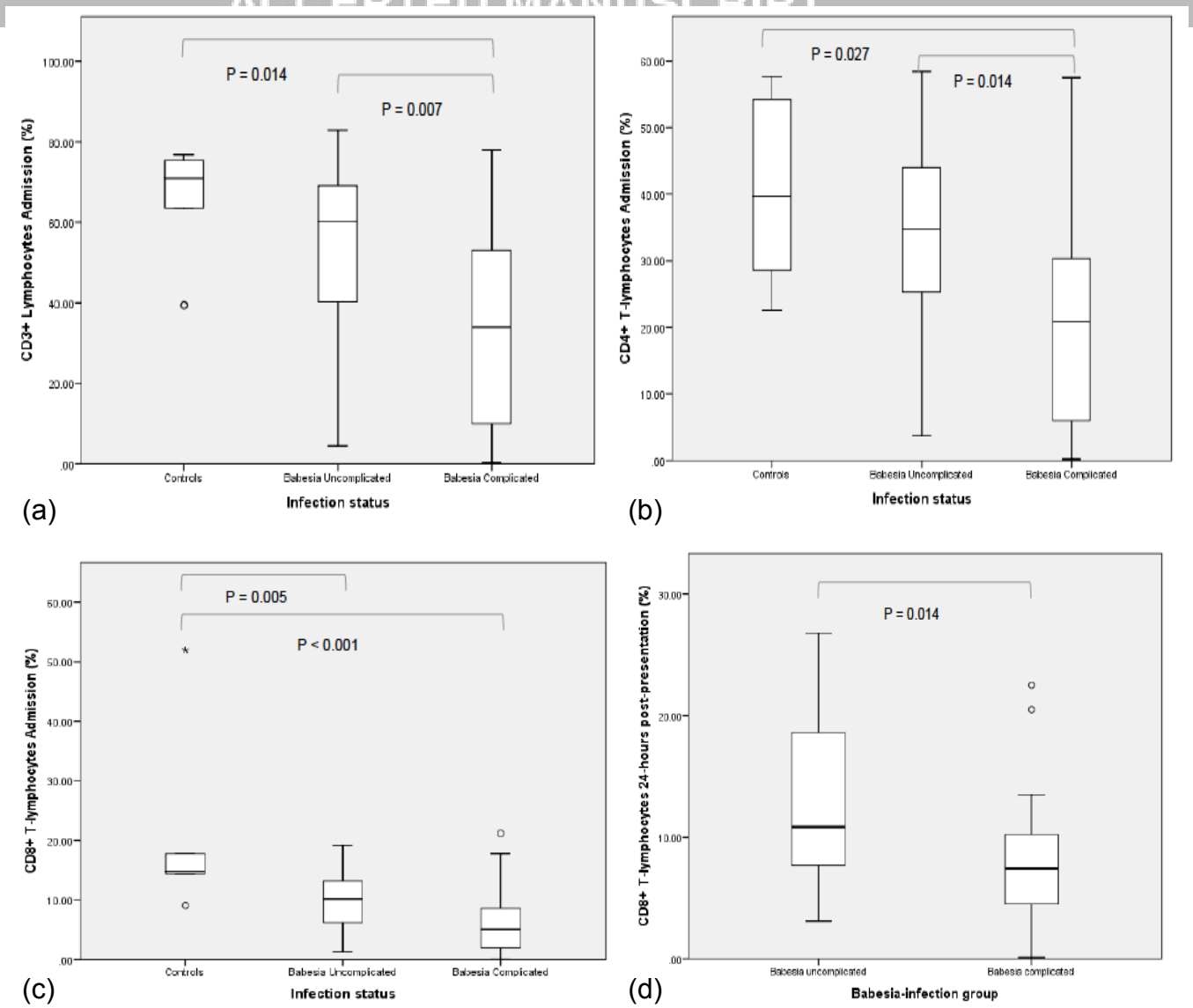

Fig. 2 Box plots of the CD3$^+$ lymphocyte (a), CD4$^+$ T lymphocyte (b),and CD8$^+$ T lymphocyte (c) percentages in t control group of dogs and the *Babesia*-infected dogs with uncomplicated and complicated disease at admission, hours post-presentation CD8$^+$ lymphocyte percentages in the *Babesia*-infected dogs with uncomplicated and co disease (d). The box represents the interquartile range (IQR), the line represents the median, the upper and low represent values within 1.5×IQR of the 75th and 25th percentiles respectively; outliers are plotted as open circles represent extreme outlier values that are 3 times removed from the IQR.